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Hypertonic Induction of Aquaporin-5 Expression through an ERK-dependent Pathway*

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Aquaporin-5 (AQP5) is a water channel protein expressed in lung, salivary gland, and lacrimal gland epithelia. Each of these sites may experience fluctuations in surface liquid osmolarity; however, osmotic regulation of AQP5 expression has not been reported. This study demonstrates that AQP5 is induced by hypertonic stress and that induction requires activation of extracellular signal-regulated kinase (ERK). Incubation of mouse lung epithelial cells (MLE-15) in hypertonic medium produced a dose-dependent increase in AQP5 expression; AQP5 protein peaked by 24 h and returned to baseline levels within hours of returning cells to isotonic medium. AQP5 induction was observed only with relatively impermeable solutes, suggesting an osmotic pressure gradient is required for induction. ERK was selectively activated in MLE-15 cells by hypertonic stress, and inhibition of ERK activation with two distinct mitogen-activated extracellular regulated kinase kinase (MEK) inhibitors, U0126 and PD98059, blocked AQP5 induction. AQP5 induction was also observed in the lung, salivary, and lacrimal glands of hyperosmolar rats, suggesting potential physiologic relevance for osmotic regulation of AQP5 expression. This report provides the first example of hypertonic induction of an extrarenal aquaporin, as well as the first association between mitogen-activated protein kinase signaling and aquaporin expression.

Aquaporin-5 (AQP5)[1] is a mammalian water channel protein that is present in the apical plasma membrane of type I pneumocytes and submucosal glands of the respiratory tract, salivary and lacrimal gland epithelia, and corneal epithelium (1–3). Aquaporins are important in the functions of these tissues. Radiation damaged salivary gland function has been partially restored by viral transfer of an aquaporin gene (4), and AQP5 knockout mice exhibit a reduction in saliva production (5). Studies of ontogeny and distribution suggest additional roles for AQP5 in lacrimation, regulation of corneal epithelial hydration, airway humidification, and generation or maintenance of the aqueous airway surface layer at multiple sites in the respiratory tract (2, 3). At each of the sites at which AQP5 is expressed, the composition or tonicity of the surface layers may be acutely altered under both normal (autonomic stimulation) and pathophysiological conditions; however, the effects of osmotic stress on AQP5 expression have not been examined.

Investigation of osmotic stress in mammalian tissues has focused principally on the kidney, in which cells of the renal medulla may experience levels >1000 mosM in humans (6). In response to these hypertonic conditions, cells accumulate small organic molecules, such as betaine, *myo*-inositol, taurine, and sorbitol. These "compatible" osmolytes lower cellular ionic strength, protecting cells from the adverse effects of elevated intracellular salt concentrations (7). Several studies have demonstrated that genes involved in the synthesis or transport of compatible osmolytes are activated by hypertonic stress (8–11). Multiple mitogen-activated protein (MAP) kinase pathways are activated by osmotic stress in cultured renal cells (12–14). Specifically, the p38 MAP kinase pathway plays a definitive role in osmotic induction of both the betaine transporter (betaine/γ-amino-*n*-butyric acid) and the sodium-dependent *myo*-inositol transporter (15, 16).

This study was designed to assess the affects of osmotic stress on AQP5 expression and to identify mechanisms regulating the response. We find that expression of AQP5 protein is induced by hypertonic stress in cultured mouse lung epithelial cells and in tissues from hyperosmolar rats. Additionally, we demonstrate that this induction requires selective activation and involvement of the ERK MAP kinase pathway. To our knowledge, these studies provide the first example of osmotic regulation of a nonrenal aquaporin and also provide the first evidence that aquaporin expression can be regulated by signaling through MAP kinase pathways.

EXPERIMENTAL PROCEDURES

Materials—Electrophoresis reagents were from Bio-Rad. Reagents for enhanced chemiluminescence (ECL+) were from Amersham Pharmacia Biotech. The BCA protein assay kit was from Pierce. TPA and PD98059 (Calbiochem) were dissolved in Me_2SO, as was U0126 (Promega, Madison, WI). Affinity-purified polyclonal antibodies to the carboxyl terminus of rat AQP5 have been described (3). Antibodies to total ERK (p44/42), total p38, phosphorylated ERK (phospho-p44/42), and phosphorylated p38 (phospho-p38) were purchased from New England Biolabs (Beverly, MA). Antibodies to total and phosphorylated JNK were from Santa Cruz Biotechnology, Inc. Antibody to Na,K-ATPase, α1-subunit was from Upstate Biotechnology (Lake Placid, NY). Horse radish peroxidase-coupled secondary antibodies specific for rabbit or mouse immunoglobulin were from Amersham Pharmacia Biotech. Except as specified, all other reagents were from Sigma.

Cell Culture and Drug Treatments—Mouse lung epithelial cells (MLE-15) (17) were a gift from Dr. Jeff Whitsett (University of Cincinnati). MLE-15 cells were grown on untreated culture dishes (Falcon/Becton Dickenson, Lincoln Park, NJ) at 37 °C with 5% CO_2 in HITES medium: RPMI 1640 medium (Life Technologies, Inc.) supplemented with insulin transferrin sodium selenite (1/10 of a stock vial/500 ml medium; Sigma), 5 μg/ml transferrin, 10 nM hydrocortisone, 10 nM

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[1] The abbreviations used are: AQP5, aquaporin-5; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; pERK, phosphorylated ERK; JNK, c-Jun NH_2-terminal kinase; MAP, mitogen-activated protein; MEK, MAP kinase/ERK kinase kinase; MLE, mouse lung epithelial; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

β-estradiol, 10 mM HEPES, 2 mM L-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2% fetal bovine serum (Life Technologies). Unless otherwise specified, medium was made hypertonic by adding 200 mosM sorbitol (36.44 mg of sorbitol/ml). In the MAP kinase inhibition studies, U0126 and PD98059 were added in the specified concentration for a 1-h preincubation before the addition of isotonic or hypertonic medium. Cells were then maintained in the medium for the duration of the experiment.

Preparation of Cell Extracts and Immunoblotting—Following the specified incubation, medium was aspirated and cells were washed in ice-cold phosphate-buffered saline prior to scraping. Scraped cells were pelleted at 10,000 × *g* for 1 min at 4 °C and resuspended in either ice-cold homogenization buffer for AQP5 immunoblotting (7.5 mM sodium phosphate, 1 mM EDTA, 1 mM sodium azide, 0.25 M sucrose, 4 μg/ml leupeptin) or phosphoprotective lysis buffer for MAP kinase immunoblotting (50 mM β-glycerophosphate (pH 7.2), 0.5% (v/v) Triton X-100, 0.1 mM sodium vanadate, 2 mM $MgCl_2$, 1 mM EGTA (pH 8.35), 1 mM dithiothreitol, 2 μg/ml leupeptin, 4 μg/ml aprotinin) (18). Cells resuspended in homogenization buffer were subjected to two rounds of freeze-thawing followed by vigorous pipeting and then spun at 800 × *g* for 5 min at 4 °C to pellet nuclei and cellular debris. Cells resuspended in phosphoprotective lysis buffer were incubated on ice for 30 min to ensure complete lysis and then pelleted at 10,000 × *g* for 10 min at 4 °C. For both homogenization protocols, total protein concentration of the sample was determined by the BCA assay using the supernatant fractions with bovine serum albumin as standard. 15–50 μg of total protein in 1.5% (w/v) SDS was loaded per lane on 12% SDS-polyacrylamide gels and subjected to SDS-PAGE using the buffer system of Laemmli (19). Duplicate gels were stained with Coomassie Brilliant Blue (Bio-Rad) to confirm equivalence of samples. Immunoblotting was performed as described (20) using enhanced chemiluminescence, and blots were visualized with autoradiography. Antibodies to Na,K-ATPase α1-subunit, as well as total and phosphorylated MAP kinases, were used according to the manufacturers' recommendations. Relative band intensities were determined by densitometry using a MacBAS bio-imaging analyzer (version 2.5, Fuji Photo Film Co.).

Northern Blots—Total RNA was isolated from MLE-15 cells using Trizol Reagent (Life Technologies). RNA concentrations were assessed spectrophotometrically. 10 μg of total RNA per sample was resolved on a formaldehyde agarose gel, transferred to nitrocellulose, and hybridized at high stringency with a full-length rat AQP5 cDNA labeled with [α-^{32}P]dCTP as described (21). Blots were visualized by autoradiography. 18 S ribosomal RNA, visualized by ultraviolet exposure of the nitrocellulose blot (Eagle Eye Systems, Stratagene, La Jolla, CA), was used as a loading control.

Animal Protocols—All animal studies were undertaken with protocols approved by the Johns Hopkins School of Medicine Animal Care and Use Committee. Male Harlan Sprague-Dawley rats (250–300 g; Harlan Sprague-Dawley, Indianapolis, IN) were used for the animal studies. Control rats were given daily intraperitoneal injections of sterile isotonic saline (150 mM NaCl; 5 ml/300 g) and allowed free access to water. Experimental rats were made hyperosmolar using a modification of previously described techniques (22); daily intraperitoneal injections of hypertonic saline (2 M NaCl; 5 ml/300 g) were given for 3 days, with water restriction for the final 24 h. All rats were euthanized by CO_2 inhalation. The thorax was opened, and an aliquot of blood was drawn directly from the right ventricle of the heart for subsequent measurement of serum osmolality by vapor pressure reduction (Wescor 5100C Vapor Pressure Osmometer). The animals were then perfused through the right and left ventricles with chilled phosphate-buffered saline until free of blood. Tissue samples were removed, frozen on dry ice, and stored at −85 °C for subsequent isolation of membranes as described previously (23). Briefly, tissues were homogenized in a Potter-Elvehjem homogenizer on ice in homogenization buffer containing 0.25 M sucrose, 1 mM EDTA, 1 mM sodium azide, 1 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml diisopropyl fluorophosphate, and 4 μg/ml leupeptin. Homogenates were centrifuged at 800 × *g* for 10 min at 4 °C, and the supernatant was then spun at 200,000 × *g* for 30 min at 4 °C. Crude membrane pellets were solubilized in 1.5% (w/v) SDS, and total protein concentration was measured. SDS-PAGE and immunoblotting were performed as described above.

Statistics—Densitometric analysis of protein immunoblots and Northern blots is expressed as mean ± S.E. ($n \geq 3$) for each group. Unpaired *t* tests were performed for some experiments to assess the effect of different interventions.

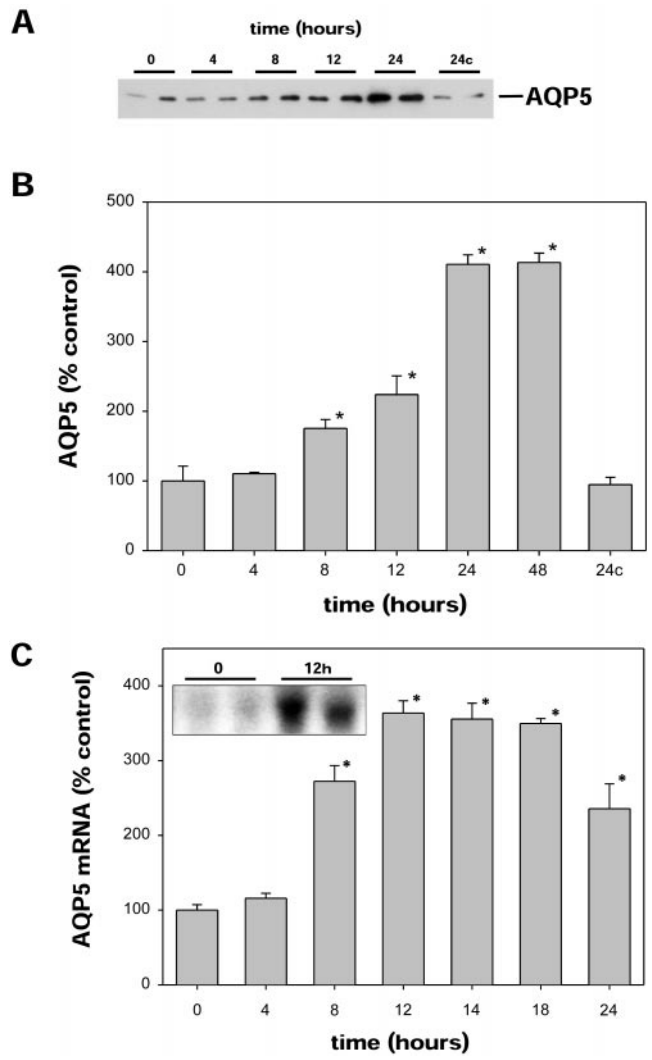

FIG. 1. **Time courses for osmotic induction of AQP5 protein and mRNA.** *A,* mouse lung epithelial (MLE-15) cells were incubated in hypertonic medium containing 200 mosM sorbitol. At the designated times (*24c* is the 24 h control sample), cells were harvested in homogenization buffer for protein immunoblot with affinity-purified anti-AQP5 antibody (anti-AQP5). *B,* protein immunoblots from cells treated as in *A* ($n = 4$ for each group) were analyzed by densitometry and are represented as a percentage of control at time 0 (mean ± S.E.; *, $p < 0.05$ *versus* time 0). *C,* MLE-15 cells were treated as in *A* and processed for Northern analysis as described. Relative band intensities (normalized to 18 S rRNA) ($n = 3$ for each group) are represented as a percentage of control at time 0 (mean ± S.E.; *, $p < 0.05$ *versus* time 0). AQP5 mRNA expression at times 0 and 12 h are shown in the *inset*.

RESULTS

AQP5 Is Induced by Hypertonic Stress in Mouse Lung Epithelial (MLE-15) Cells—Mouse lung epithelial cells (MLE-15) were incubated in normal medium supplemented with 200 mosM sorbitol (final concentration, 500 mosM), and samples were harvested at specified times for immunoblot and Northern analysis. AQP5 protein increased by 8 h after exposure to hypertonic medium and peaked at 24 h (Fig. 1, *A* and *B*), whereas AQP5 mRNA peaked by 12 h (Fig. 1*C*). Following an initial induction by hypertonic medium (24 h), AQP5 protein expression was reduced nearly to baseline levels within 6 h after returning cells to isotonic medium (Fig. 2*A, top panel*); AQP5 protein levels continued to be elevated in cells remaining in hypertonic conditions (Fig. 2*A, bottom panel*). Similarly, following a 12 h hypertonic induction, AQP5 mRNA was markedly reduced within 2 h after cells were returned to isotonic medium (Fig. 2, *B* and *C, lane 2*), as compared with mRNA

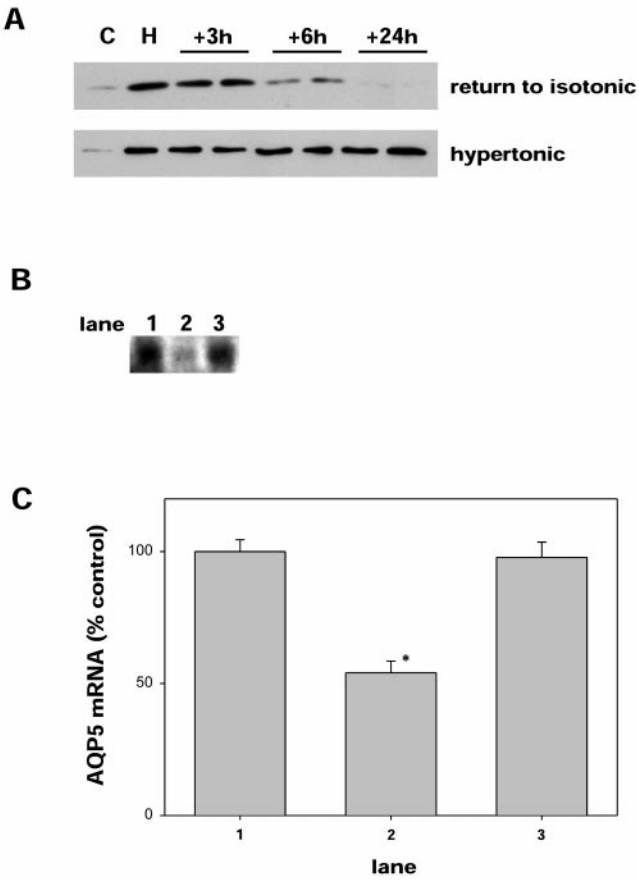

FIG. 2. **AQP5 expression decreases when cells are returned to isotonic medium.** *A,* cells were incubated in control (*C*) or hypertonic (*H*) medium for 24 h and then returned to isotonic medium (*top panel*) or maintained in hypertonic medium (*bottom panel*) for another 3, 6, or 24 h prior to immunoblot analysis. *B,* Northern blot of cells incubated in hypertonic medium for 12 h (*lane 1*), hypertonic medium for 12 h and returned to isotonic for 2 h (*lane 2*), and hypertonic medium for 14 h (*lane 3*). *C,* Northern blots from cells treated as in *B* ($n = 3$ for each group) were analyzed by densitometry and expressed as a percentage of the mean 12 h time point (mean ± S.E.; *, $p < 0.05$ *versus* 12 h).

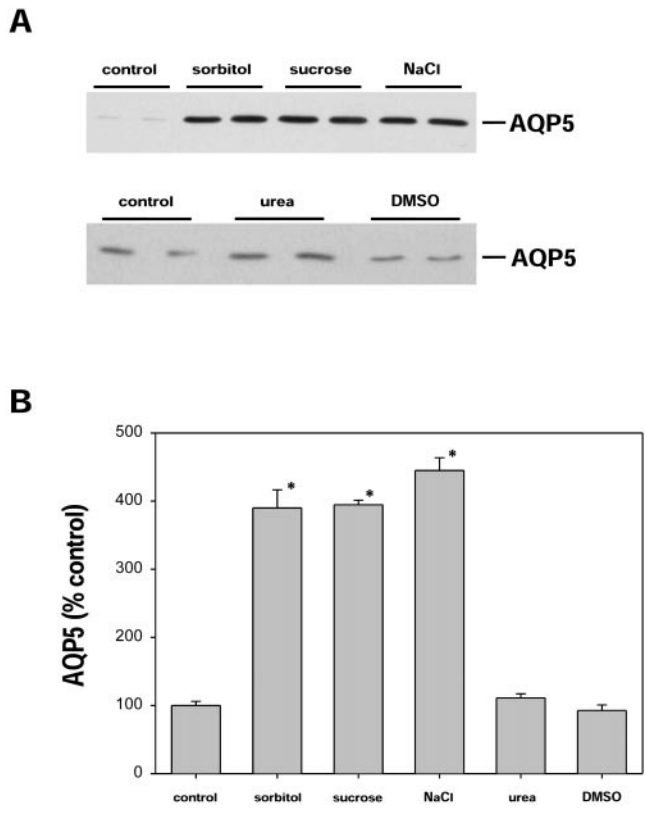

FIG. 3. **Hypertonic induction of AQP5 by different solutes.** *A,* MLE-15 cells were incubated in control medium or medium with 200 mosM of ionic (NaCl) and nonionic (sorbitol, sucrose, urea, and Me_2SO (*DMSO*)) osmolytes for 18–20 h and then processed for immunoblot analysis as described. *B,* protein immunoblots from cells treated as in *A* ($n = 4$ for each group) were analyzed by densitometry and expressed as a percentage of isotonic control (mean ± S.E.; *, $p < 0.05$ *versus* control).

expression in cells exposed to hypertonic stress for 12 and 14 h (Fig. 2, *B* and *C, lanes 1* and *3*). To determine whether hypertonic induction of AQP5 was solute-specific, we incubated MLE-15 cells in medium supplemented with 200 mosM ionic (NaCl) and nonionic (sorbitol, sucrose, urea, and Me_2SO) solutes. AQP5 protein expression was only increased by relatively impermeable solutes (sorbitol, sucrose, and NaCl), whereas permeable solutes, such as urea and Me_2SO, had little effect on protein expression (Fig. 3). These data suggest that a hypertonic stimulus (osmotic gradient), not simply hyperosmolarity (increased solute content), is needed for AQP5 induction.

Dose-response experiments revealed that AQP5 protein was induced maximally when MLE-15 cells were incubated in medium supplemented with 200 mosM sorbitol (Fig. 4, *A* and *B*). AQP5 was induced to a lesser extent by addition of 300 mosM sorbitol; however, cell viability was diminished at that level of osmotic stress. To test potential induction of AQP5 by more subtle changes in medium osmolarity, MLE-15 cells were incubated in medium with 25, 50, and 100 mosM sorbitol (Fig. 4, *C* and *D*). Addition of 25 mosM sorbitol was sufficient to increase AQP5 protein expression over that of isotonic controls, and greater induction was observed as medium osmolarity increased.

AQP5 mRNA Is Not Significantly Stabilized by Hypertonic Stress—To determine whether stabilization of AQP5 mRNA accounts for osmotic induction of AQP5 by hypertonicity, cells incubated in isotonic or hypertonic medium for 12 h were treated with the transcriptional inhibitor actinomycin D (5 μg/ml) for the designated times and processed for Northern blotting (Fig. 5*A*). Although the overall abundance of AQP5 mRNA was increased after incubation in hypertonic medium, hypertonicity did not stabilize the AQP5 mRNA (normalized to 18 S rRNA) compared with isotonic controls (Fig. 5*B*; mean of three trials).

ERK Is Activated by Hypertonic Stress in MLE-15 Cells—Previous reports have shown that all three MAP kinases (ERK, JNK, and p38) can be activated by osmotic stress (12–14). In particular, p38 has frequently been implicated in the induction of genes involved in organic osmolyte synthesis and transport (15, 16). Therefore, we wanted to determine whether MAP kinase-mediated signaling was involved in hypertonic induction of AQP5 in MLE-15 cells. Utilizing antibodies specific for either the activated (phosphorylated) or total form of each of the three MAP kinases (ERK, JNK, and p38), we performed immunoblot analysis on cells incubated in isotonic or hypertonic medium (Fig. 6*A*). ERK was selectively activated by hypertonic stress in MLE-15 cells; phosphorylated ERK (pERK) peaked 15 min after hypertonic exposure, increasing by approximately 4-fold (Fig. 6). The total amount of ERK remained constant, as did the amounts of both phosphorylated and total JNK and p38 (Fig. 6). Time points were selected based on previous reports showing activation of MAP kinases within the first hour after the initial stimulus (24–27).

Specific ERK Pathway Inhibitors Prevent Hypertonic Induction of AQP5—Because hypertonic stress activated the ERK pathway but not the JNK or p38 MAP kinase pathways in MLE-15 cells, we examined the effects of the MEK1/2 inhibitors U0126 and PD98059 on AQP5 induction. MEK1/2 are the upstream kinases that activate ERK. Both U0126 and

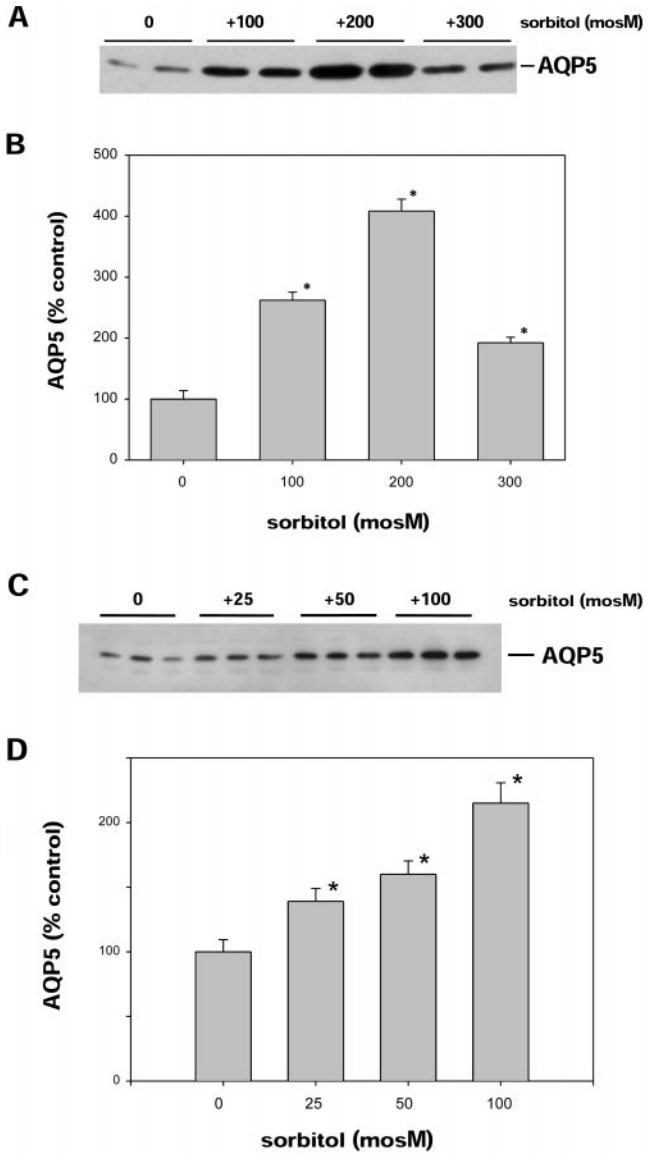

FIG. 4. **Dose response for hypertonic induction of AQP5.** *A,* MLE-15 cells were incubated in isotonic or hypertonic medium (supplemented with sorbitol as noted) for 20 h and then harvested for immunoblot analysis with anti-AQP5 antibody. *B,* protein immunoblots from cells treated as in *A* ($n = 3$ for each group) were analyzed by densitometry and represented as a percentage of isotonic control (mean ± S.E.; *, $p < 0.05$ *versus* control). *C,* MLE-15 cells were incubated in medium supplemented with sorbitol as indicated and harvested for immunoblot analysis with anti-AQP5 antibody. *D,* relative intensities of the bands in *C* are represented as percentages of control (mean ± S.E.; $n = 3$ per group; *, $p < 0.05$ *versus* control).

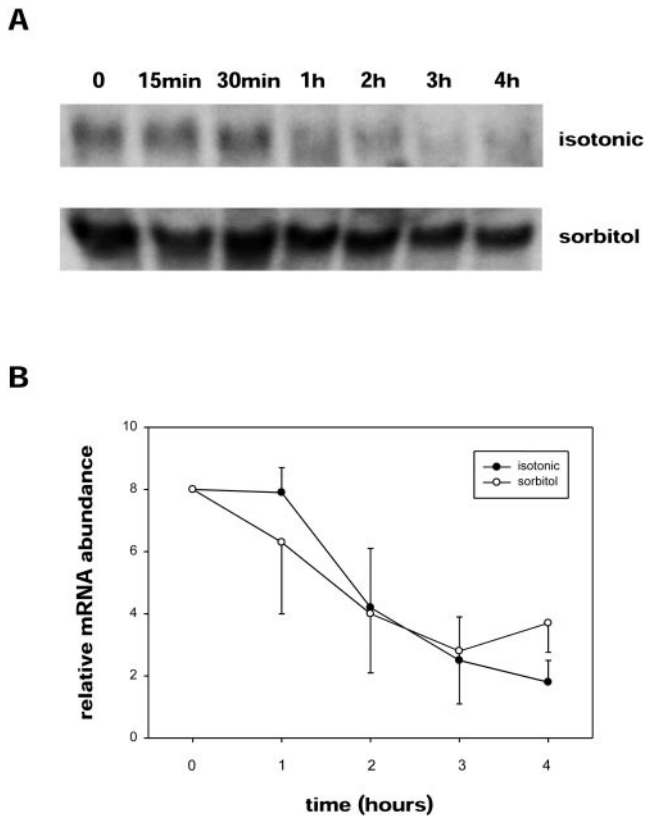

FIG. 5. **AQP5 mRNA is not significantly stabilized by hypertonic stress.** *A,* cells were incubated in either isotonic (*top panel*) or hypertonic medium (*bottom panel*) for 12 h. Actinomycin D (5 μg/ml) was then added, and cells were harvested at the designated time points for Northern analysis. *B,* relative band intensities for isotonic and hypertonic cells from three separate RNA stability trials were quantitated by densitometry, normalized to the 18 S ribosomal RNA signal for each sample, and represented as a percentage of the baseline expression for each group at the indicated times (mean ± S.E.; $n = 3$).

PD98059 inhibit MEK1/2; however, their binding affinities and mechanism of action are distinct (28). U0126 inhibits the kinase activity of MEK1/2, whereas PD98059 blocks activation of MEK1/2 by Raf kinase. In MLE-15 cells, U0126 and PD98059 both inhibited basal and hypertonicity-induced phosphorylation of ERK (Fig. 7). U0126 was a more potent inhibitor of ERK activation, consistent with prior reports (28). Incubation of MLE-15 cells with either 10 or 50 μM U0126 reduced AQP5 protein induction by hypertonicity but had no effect on basal AQP5 expression (Fig. 8, *A* and *B*). Hypertonic induction of AQP5 protein was similarly blocked by PD98059 (Fig. 8*A, bottom panel*). Incubation of cells with U0126 and PD98059 (50 μM) also reduced hypertonic induction of AQP5 mRNA (Fig. 8*C*), suggesting potential inhibition at the level of transcription. To determine whether the MEK inhibitors were acting specifically to prevent AQP5 induction, we incubated cells with U0126 as stated above and probed for Na,K-ATPase (Fig. 8, *D* and *E*), another integral membrane protein known to be induced by hypertonicity (29, 30). In MLE-15 cells, the α-subunit of Na,K-ATPase was induced nearly 2-fold by hypertonic stress (Fig. 8, *D* and *E*). In contrast to the inhibition of AQP5 induction, U0126 did not affect expression of Na,K-ATPase, suggesting that reduction of AQP5 by the MEK inhibitors was not a result of nonspecific effects on total protein synthesis.

ERK Activation Is Necessary but Not Sufficient for AQP5 Induction by Hypertonic Stress—The studies above suggest that ERK activation is necessary for AQP5 induction by hypertonic stress. To determine whether ERK activation is sufficient for AQP5 induction, MLE-15 cells were incubated with TPA (100 nM) under isotonic and hypertonic conditions. TPA is a potent activator of both protein kinase C and ERK (31). Addition of TPA to the medium activated ERK (Fig. 9*A*) but did not increase basal AQP5 expression, nor did it augment the induction of AQP5 by hypertonicity (Fig. 9*B*). Similar results were seen when 100 nM TPA was incubated with MLE-15 cells for only 1 h (data not shown), suggesting that the previous findings were not confounded by chronic inhibition of protein kinase C expression by TPA (12, 32). These results suggest that ERK activation is necessary but not sufficient for hypertonic induction of AQP5.

AQP5 Expression Is Induced in Rat Tissues by Hyperosmolarity—To investigate whether hypertonic induction of AQP5 can occur *in vivo*, AQP5 expression was determined in control and hyperosmolar rats. As compared with control rats (mean serum osmolality, 310 ± 2.3 milliosmoles/kg; $n = 4$), hypertonic animals

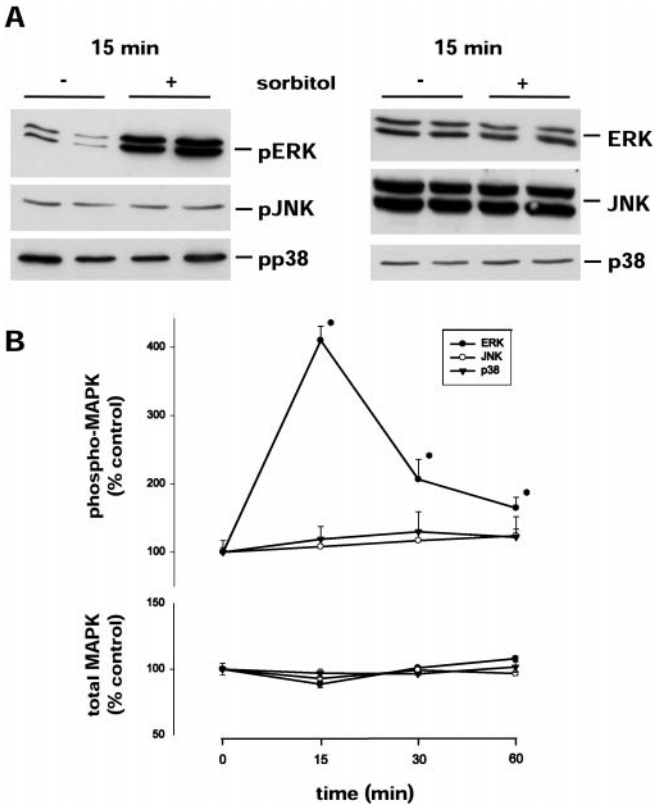

FIG. 6. **ERK is selectively activated relative to other MAP kinases under osmotic stress.** *A,* MLE-15 cells were exposed to control or hypertonic medium for 15 min and then harvested in phosphoprotective buffer. Protein immunoblots were performed using antibody to either phosphorylated (*pERK, pJNK,* and *pp38*) or total (*ERK, JNK,* and *p38*) MAP kinase. *B,* MLE-15 cells were incubated in hypertonic medium for the designated time and processed as in *A* for either phosphorylated (*top panel*) or total (*bottom panel*) MAP kinase. Immunoblots were analyzed by densitometry and expressed as a percentage of control for each MAP kinase (mean ± S.E.; $n = 4$; *, $p < 0.05$ *versus* time 0).

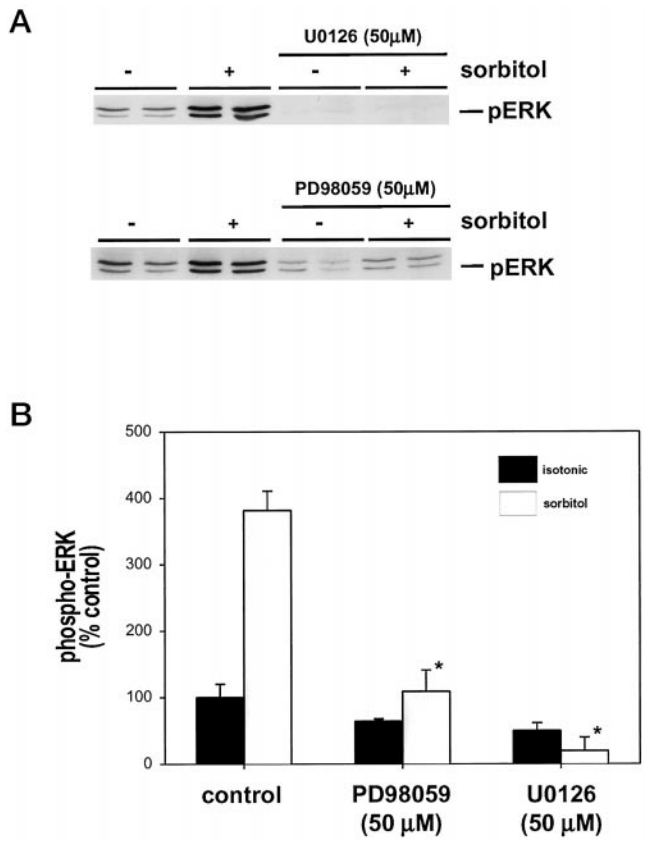

FIG. 7. **Specific ERK pathway inhibitors prevent both basal and hypertonicity-induced phosphorylation of ERK.** *A,* MLE-15 cells were treated with isotonic or hypertonic medium for 15 min in the presence or absence of either U0126 or PD98059 (50 μM) and harvested in phosphoprotective buffer for immunoblot with anti-pERK antibody. *B,* protein immunoblots from cells treated as in *A* ($n = 3$ for each group) were analyzed by densitometry and expressed as a percentage of isotonic control (mean ± S.E.; *, $p < 0.05$ *versus* hypertonic control).

(mean, 335 ± 2.0 milliosmoles/kg; $n = 4$) had increased expression of AQP5 in lung (2-fold), lacrimal gland (7-fold), and submandibular gland (2-fold) (Fig. 10). These data demonstrate that hypertonic induction of AQP5 is not an isolated *in vitro* phenomenon.

DISCUSSION

Discovery of the aquaporin family of water channel proteins has provided insight into molecular mechanisms of membrane water permeability. It is increasingly clear that aquaporins can be rate-limiting for water transport, as evidenced by recent demonstrations in the kidney (33, 34), lung (35), and salivary glands (5). However, with few exceptions, mechanisms regulating aquaporin expression remain poorly understood. These studies were undertaken to examine the effects of osmotic stress on expression of AQP5, an epithelial water channel found in the apical membrane of type I pneumocytes and submucosal glands in the respiratory tract, as well as in the apical membrane of salivary and lacrimal glands and corneal epithelium. In each of these locations, the osmolality of aqueous surface layers may vary in some circumstances. Here, we describe that in a lung epithelial cell line, as well as in rat tissues, AQP5 is up-regulated by hypertonic stress. Osmotic induction of AQP1 in a kidney cell line was recently described (36). The studies reported here provide the first example of osmotic induction of a nonrenal aquaporin, as well as the first demonstration that aquaporin expression can be regulated by the MAP kinase signaling cascade.

Upon exposure to hyperosmotic stress, inorganic ions flow into cells, only to be replaced over several hours by organic osmolytes, such as betaine and *myo*-inositol (37). Although total DNA synthesis, RNA transcription, and protein synthesis decrease with osmotic stress (38), a limited number of genes have been identified that are up-regulated (37, 39). Induction of the transporters and enzymes that facilitate accumulation of intracellular organic osmolytes has been the focus of intense investigation (37). Given the intimate relationship of solute and water transport, we explored whether aquaporin expression might also be affected by osmotic stress. AQP5 mRNA and protein were induced by hypertonic stress in MLE-15 cells. AQP5 mRNA expression peaked at 12 h, whereas protein expression peaked at 24 h, a time course similar to induction of organic solute transporters (40). When MLE-15 cells were incubated with hypertonic medium and then returned to isotonic conditions, AQP5 mRNA and protein expression fell nearly to baseline levels within hours, indicating that both expression and degradation may be tightly controlled.

These studies provide the first link between aquaporin expression and a MAP kinase signaling cascade. MAP kinases have been implicated in a wide range of cellular events, from growth factor-mediated proliferation to numerous stress responses (41, 42). We demonstrate selective activation of ERK by hypertonic stress in MLE-15 cells. This finding contrasts to previous studies in alveolar type II cells (31) and inner medullary collecting duct cells (13, 14), in which all three MAP kinase cascades (ERK, JNK, and p38) are activated by osmotic stress. Incubation of cells with two distinct MEK inhibitors, U0126 and PD98059, prevented activation of ERK by hypertonic

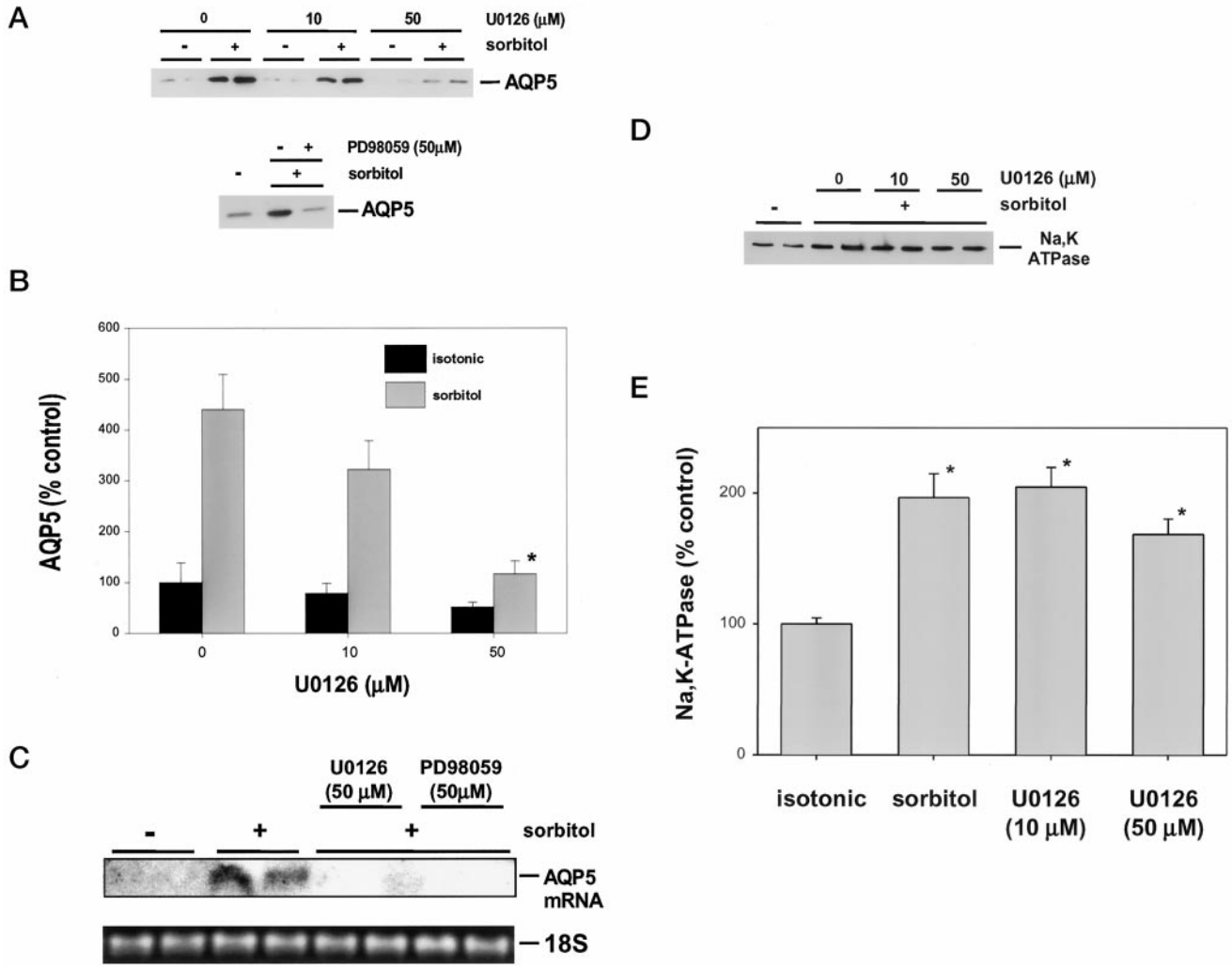

FIG. 8. **ERK pathway inhibitors prevent hypertonic induction of AQP5.** *A,* MLE-15 cells were incubated in isotonic or hypertonic medium for 18–20 h in the presence or absence of the ERK inhibitors U0126 (10 or 50 μM) or PD98059 (50 μM). Cells were harvested in homogenization buffer, and protein immunoblots were probed with anti-AQP5. *B,* protein immunoblots from cells treated as in *A* ($n = 4$ each group) were analyzed by densitometry and expressed as a percentage of control (mean ± S.E.; *, $p < 0.05$ *versus* hypertonic control). *C,* cells were treated with U0126 or PD98059 (50 μM) in the presence of hypertonic medium for 12 h and processed for Northern analysis (*top panel*) with the full-length rat AQP5 cDNA. 18 S ribosomal RNA was utilized as a loading control (*bottom panel*). *D,* cells were treated with U0126 as in *A,* and the immunoblot was probed for the α-subunit of Na,K-ATPase. *E,* protein immunoblots from cells treated as in *D* ($n = 4$ for each group) were analyzed by densitometry and expressed as a percentage of isotonic control (mean ± S.E.; *, $p < 0.05$ *versus* control).

stress and dramatically blocked both AQP5 mRNA and protein induction. Neither basal nor hypertonically induced Na,K-ATPase expression was affected by these drugs, suggesting that these inhibitors are not globally affecting protein synthesis pathways.

Although ERK activation is necessary for AQP5 induction in this model, activation of ERK by TPA did not increase AQP5 expression, either at baseline or following hypertonic stimulation, indicating that additional signaling steps are required. Signaling events both upstream and downstream from ERK in this model are currently under investigation. AQP5 was induced only when relatively impermeable solutes were added to the medium. This requirement for hypertonic, rather than simply hyperosmolar, stimulation provides some insight into upstream events, as it suggests that perturbation of the cell membrane or cytoskeleton may be necessary for induction to occur. Downstream ERK is known to activate several transcription factors, including Elk1, Ets1, c-Myc, Tal, and STAT, which then directly activate target genes (42). Our data suggest transcriptional activation of AQP5, because hypertonic stress increased AQP5 mRNA expression severalfold without increasing mRNA stability. This effect may not be direct, however, based both on the time course of induction (several hours required) and preliminary studies showing that cyclohexamide blocks hypertonic induction of AQP5 mRNA (data not shown). A recently identified hypertonicity-induced transcription factor, TonEBP (tonicity-responsive element-binding protein) (43), is a candidate protein for the intermediate in this pathway. We have been unable to demonstrate activation of a 1.5-kilobase AQP5 proximal promoter by hypertonicity in preliminary experiments (data not shown); however, no tonicity response element consensus sites are present in this part of the promoter. Tonicity response elements have been identified as far as 50 kilobases upstream in the case of sodium-dependent *myo*-inositol transporter (44). We suspect that, in a similar fashion, relevant *cis* elements in the AQP5 promoter are outside of the 1.5-kilobase proximal promoter as well.

We believe that these data strongly support potential *in vivo* relevance of this phenomenon for two reasons. First, surface liquid osmolalities have been demonstrated under some circumstances to reach 430–480 milliosmoles/kg in human nasal secretions (45) and canine airway secretions (46, 47). We observed a dose-response relationship for hypertonic induction of AQP5 beginning with as little as 25 mosM supplemental sorbi-

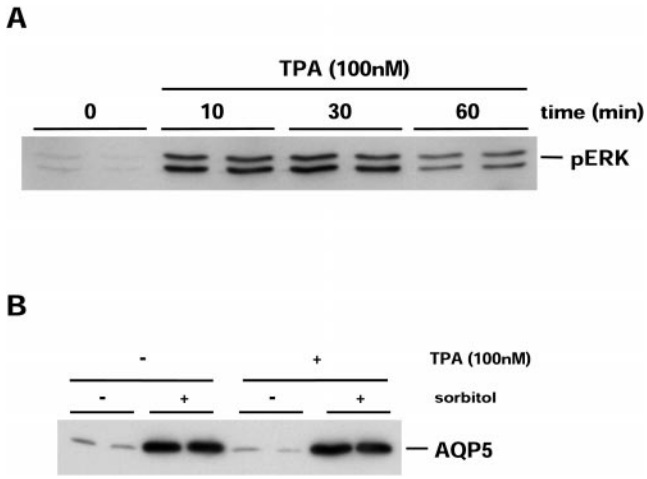

FIG. 9. **ERK activation is not sufficient for AQP5 induction.** *A,* MLE-15 cells were incubated with 100 nM TPA in isotonic medium for the designated times, harvested in phosphoprotective buffer, and immunoblotted with anti-pERK antibody. *B,* cells were incubated in control or hypertonic medium in the presence or absence of TPA (100 nM) for 18 h and harvested in homogenization buffer, and immunoblots were probed with anti-AQP5 antibody.

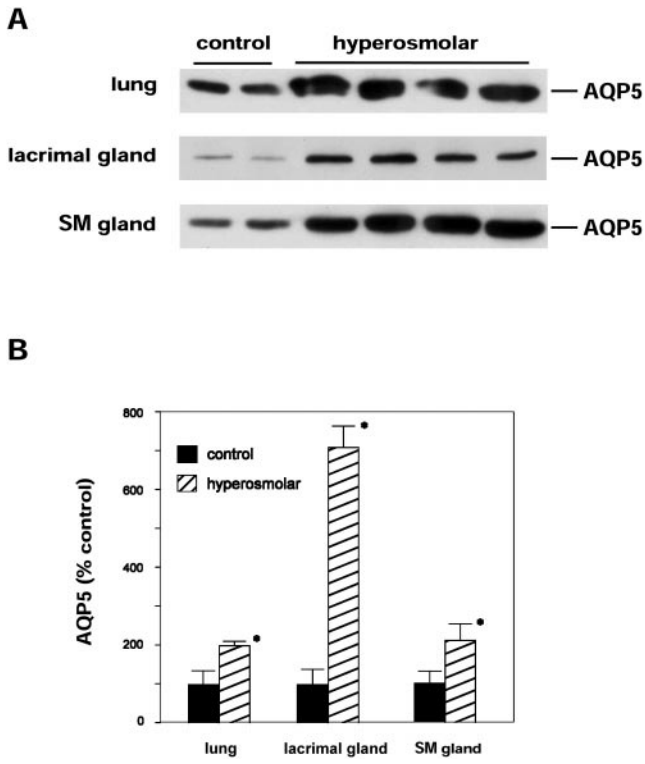

FIG. 10. ***In vivo* osmotic induction of AQP5 in rat tissues.** *A,* control rats were given daily intraperitoneal injections of isotonic saline (1.5 mM NaCl; 5 ml/300 g) and given water *ad libitum*. Hyperosmolar rats were given daily intraperitoneal injections of hypertonic saline (2 M NaCl; 5 ml/300 g) for 3 days and deprived of water for the final 24 h. Samples of lung, lacrimal gland, and submandibular (*SM*) gland were isolated, processed for protein immunoblotting as described, and probed with anti-AQP5 antibody. *B,* immunoblots were analyzed by densitometry and expressed as percentages of control (mean ± S.E.; $n = 4$ each group; *, $p < 0.05$ *versus* control).

tol, well within this range. Second, and more importantly, using previously described techniques for generating hyperosmolarity *in vivo* (22), we show that AQP5 was induced in the lung, submandibular gland, and lacrimal gland of hyperosmolar rats when compared with isosmolar controls. Future investigation of this phenomenon in additional *in vivo* models will allow more specific assessment of the role for hypertonic induction of AQP5 in different pathophysiologic conditions.

With the recognition that aquaporins can be rate-limiting for water transport comes an increased need for understanding the mechanisms regulating their expression, as well as their relevance to human disease. Changes in the tonicity of airway surface liquid have been implicated in the pathophysiology of cystic fibrosis (48), as well as in some forms of exercise- or cold-induced asthma (45, 49, 50). Conditions associated with increased minute ventilation have been shown to increase airway drying and alter surface liquid osmolarity. Likewise, dry eyes and mouth are common clinical problems; in some patients the gland dysfunction is immunologically mediated (Sjogren's syndrome), although in most, it is not. In all of the above circumstances, dynamic regulation of AQP5 expression could be an appropriate response to changes in surface liquid tonicity, perhaps as a mechanism for restoring lumenal osmolarity or maintaining cell volume. Additionally, identification of ERK as a necessary signaling component in this model provides the first example linking aquaporin expression to intracellular signaling pathways. Involvement of ERK or other MAP kinases will likely extend to other models in which aquaporin expression may be altered, for example with inflammation or oxidant stress. Identification of both the stimuli and signaling pathways that regulate aquaporin expression may provide insight into alterations in water transport seen in a wide array of pathophysiologic conditions.

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